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Accelerated Publications

The C-Terminus of Tissue Factor Pathway Inhibitor Is Essential to Its Anticoagulant Activity

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ABSTRACT: Tissue factor pathway inhibitor (TFPI) from different cell lines shows up to 15-fold differences in the ratio of anticoagulant to chromogenic activity. The anticoagulant activity was dependent on the purification procedure used and it was possible to isolate two fractions of recombinant TFPI. Only one of these fractions showed anticoagulant activity comparable with TFPI from normal human plasma, and Western blotting showed that the low-activity fraction did not react with an antibody raised against a peptide of TFPI located near the C-terminal. Analysis by mass spectroscopy of peptides from V8 protease digests showed that C-terminal amino acids could only be identified from the high-activity form, while heterologous fragmentation had taken place in the form with low anticoagulant activity. Previously published studies on TFPI have been performed using material of low anticoagulant activity compared with plasma TFPI, and we suggest that these studies have been performed with material degraded in the C-terminus.

The tissue factor dependent pathway of coagulation is inhibited by tissue factor pathway inhibitor (TFPI),¹ which until recently was called extrinsic pathway inhibitor (EPI) or lipoprotein-associated coagulation inhibitor (LACI) (Rapaport, 1989; Broze et al., 1990). TFPI is a 42-kDa glycoprotein consisting of three tandem Kunitz-type inhibitor domains, a negatively charged N-terminus, and a positively charged C-terminus (Wun et al., 1988). The second Kunitz domain of TFPI binds and inhibits factor Xa (Warn-Cramer et al., 1988; Broze et al., 1988), and the TFPI-factor Xa complex inhibits factor VIIa-tissue factor in a calcium-dependent manner. By site-directed mutagenesis it has been shown that it is the first Kunitz domain of TFPI that is responsible for the inhibition of factor VIIa (Girard et al., 1989).

In normal human plasma, TFPI is in complex with lipoproteins (Novotny et al., 1989). Sandset et al. (1988) have shown that heparin injection results in an increased plasma concentration of TFPI, probably due to release from endothelial cells. This heparin-released TFPI prolongs the coagulation time of tissue factor dependent clotting assays when coagulation is initiated by relatively small amounts of tissue factor (Lindahl et al., 1991). When compared on the basis of TFPI activity measured by a chromogenic two-stage assay, the TFPI in normal plasma and the TFPI released by heparin have a higher anticoagulant activity than TFPI prepared by recombinant DNA technology (Nordfang et al., 1991; Lindahl et al., 1991). TFPI that was partly purified from plasma by one-step affinity chromatography was observed to lose its anticoagulant activity with a half-life of 1 h at room temperature, while the chromogenic activity of TFPI was hardly affected (Valentin et al., 1991).

Many human cell lines produce proteins with TFPI-like activity (Bajaj et al., 1987; Wun et al., 1990). Since only 1 ppm of the protein in human plasma is TFPI, most studies with TFPI have been carried out using TFPI isolated from the culture media of some of these cell lines (Broze & Miletich,

1987; Warn-Cramer et al., 1989). Wun et al. (1990) showed that the anticoagulant activity of TFPI originating from various cell lines differs, and it was found that the most active forms have an activity corresponding to that of plasma TFPI. It was suggested that a posttranslational modification might be responsible when full anticoagulant activity of TFPI was obtained. We have now sought to elucidate the reason for the variation in anticoagulant activity between the different forms of TFPI, and the present paper describes the structural characterization of two molecular forms of rTFPI.

MATERIALS AND METHODS

Proteins. Recombinant TFPI was purified from the culture media of transfected BHK cells as described previously (Pedersen et al., 1990). The purification procedure consisted of four steps: (1) heparin affinity chromatography, (2) Mono Q anion exchange, (3) Mono S cation exchange, and (4) RP-HPLC. When analyzed in the chromogenic TFPI activity assay, the purified rTFPI exhibited a specific activity of 30 000 units/mg and the anticoagulant activity corresponded to that previously reported for rTFPI isolated from C127 cells (Day et al., 1990). Thus the PT coagulation time for human plasma using partly diluted rabbit tissue thromboplastin was prolonged from 35 to 65 s by the addition of 2 µg/mL rTFPI. TFPI from human plasma was partly purified using a one-step affinity chromatography procedure (Valentin et al., 1991). The specific activity of the TFPI preparation was 100 units/mg when measured in the chromogenic assay. For freshly prepared pTFPI the ratio of anticoagulant to chromogenic activity corresponded to that of unfractionated human plasma.

A polyclonal antibody inhibiting TFPI was obtained by immunizing rabbits with rTFPI. In coagulation assays this antibody shortens the clotting time solely by inhibiting TFPI (Nordfang et al., 1991). An antibody directed toward the

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positively charged region near the C-terminus of TFPI was obtained by immunizing with a synthetic peptide deduced from the cDNA sequence (Wun et al., 1988). Three milligrams of synthetic peptide (Thr Lys Arg Lys Arg Lys Lys Gln Arg) was coupled to diphtheria toxin (1:1 w/w) with 5% glutaraldehyde in 0.3 M NaCl, pH 7.0. Following coupling, excess glutaraldehyde was removed by dialysis. Rabbits were given four doses of coupled peptide (equivalent to 100 µg of peptide) in Freund's adjuvant at 28-day intervals.

Assays. TFPI activity was measured in a chromogenic two-stage assay developed by Sandset et al. (1987). This assay was adapted for microplates as described by Pedersen et al. (1990). The standard was a pool of normal human plasma (1 unit/mL TFPI). The anticoagulant activity of purified TFPI preparations was assessed in a PT coagulation assay using dilute human tissue thromboplastin as activator. A pool of normal human plasma was mixed with either one-tenth volume of TFPI, an excess of inhibiting anti-TFPI IgG, or control buffer. Following a 15-min incubation at room temperature, 75 µL of the incubation mixture was mixed with 75 µL of thromboplastin/CaCl₂ reagent and the coagulation time was registered on an ACL coagulation apparatus equipped with a computer to enable measurement of long clotting times. The thromboplastin/CaCl₂ reagent contained 17 mM imidazole, 20 mM CaCl₂, 0.33 M NaCl, 0.33 mg/mL bovine serum albumin, and human tissue thromboplastin (Nawroth et al., 1986) diluted 15000-fold. In this system the clotting time of the normal human plasma was approximately 100 s and antibody toward TFPI shortened the clotting time by approximately 30 s. Samples prolonging the clotting time with an equivalent number of seconds were set to contain 1 unit/mL of anticoagulant activity.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970), using 12.5% polyacrylamide separating gels unless otherwise indicated. Following electrophoresis, the proteins were subjected to Western blotting (Mikkelsen & Knudsen, 1987). Proteins were stained with Auro Dye from Amersham or immunodetected by rabbit antibodies and phosphatase-labeled secondary anti-rabbit IgG from Dakopatts.

Cell Culturing. The BHK cell line producing rTFPI was grown in fermentors as described previously (Pedersen et al., 1990). HeLa larynx carcinoma and HepG2 hepatoma cells were grown to confluence in DMEM with 10% fetal calf serum. The cells were then washed three times with serum-free medium in the T-flasks and incubated for 4 days in DMEM with additives as indicated in Table III. Culture medium was harvested and stored frozen until analysis and purification.

Protein Purification. Unless otherwise stated, rTFPI and pTFPI were purified as described above. For the experiment shown in Table III, TFPI was purified from the culture medium by a one-step affinity chromatography procedure. In this procedure 25 mL of culture medium was incubated for 1 h at room temperature with 0.5 mL of Sepharose conjugated with 5 mg/mL inhibiting polyclonal anti-TFPI IgG. After an initial wash with 10 mL of TBS (50 mM Tris and 0.1 M NaCl, pH 7.4), TFPI was eluted with 2 mL of 0.1 M glycine hydrochloride, pH 2.5. The eluates were kept at -20 °C until analysis.

Mono-S separation of rTFPI forms was obtained after purification on heparin-Sepharose and Mono Q. Fractions obtained from the Mono Q column were diluted 5-fold in buffer A (20 mM sodium citrate and 10% glycerol, pH 5.0) and applied on a Mono S HR 5/5 column at a flow rate of 0.4 mL/min. After a brief wash with buffer A, TFPI was eluted

in a linear gradient from buffer A to 50 mM imidazole hydrochloride, 0.6 M NaCl, and 10% glycerol, pH 7.4. As a final purification step, the two TFPI-containing peaks eluted from the Mono S column were subjected to reverse-phase chromatography on a Pro RPC 5/10 column as described previously (Pedersen et al., 1990).

Structural Characterization of rTFPI. (A) *Derivatization.* Approximately 100 µg of rTFPI with high [Figure 1 (bottom), peaks X + Y] or low [Figure 1 (middle), peak X] anticoagulant activity was reduced by incubation at 37 °C for 2 hours in 200 µL of 0.2 M Tris and 6 M guanidine hydrochloride, pH 8.0, containing 2 mg of dithiothreitol. Pyridylethylation of the free SH groups was obtained by addition of 6 µL of 4-vinylpyridine and incubation at room temperature for 30 min. The alkylation reaction was stopped by adding 16 mg of dithiothreitol. The TFPI derivatives were purified by reverse-phase HPLC on a Vydac 214TP54 column using 0.1% TFA as eluent A and 0.07% TFA in acetonitrile as eluent B. Equilibration was performed with 5% eluent B at room temperature at a flow rate of 1.5 mL/min. A linear gradient from 5 to 65% eluent B was pumped through the column. Fractions containing the pyridylethylated TFPI were collected and lyophilized in a vacuum centrifuge.

(B) *Peptide Mapping.* Approximately 20 µg of the TFPI derivatives were dissolved in 150 µL of 50 mM ammonium acetate, pH 4.0, and digested with 1 µg of V8 protease at 37 °C for 16 h. Peptide mapping was performed using the column and eluents described above. Equilibration was performed with 100% eluent A at ambient temperature at a flow rate of 1.5 mL/min. A linear gradient from 0 to 40% eluent B was pumped through the column and collection of the effluent in fractions was performed manually based on the absorbance at 214 nm. The fractions were lyophilized in a vacuum centrifuge, and the material was redissolved in 50 µL of 0.1% TFA and 20% ethanol before aliquots were analyzed by mass spectrometry and/or amino acid sequencing.

(C) *Mass Spectrometry.* Molecular weight determination was obtained on a Bio-Ion 20 plasma desorption mass spectrometer (PDMS) equipped and operated in positive mode with a flight tube of approximately 15 cm as previously described (Bjorn et al., 1991). Aliquots of 5 µL were analyzed at an accelerating voltage set to 15 kV and positive ions were collected for 5 million fission events. The accuracy on assigned molecular ions is approximately 0.1% for well-defined peaks, otherwise somewhat less (Roepstorff, 1989).

(D) *Amino Acid Sequencing.* N-Terminal sequence analysis was carried out by automated Edman degradation using an Applied Biosystems 470 A gas-phase sequencer. Analysis by on-line reverse-phase HPLC was performed for the detection and quantification of the liberated PTH amino acids from each sequence cycle.

RESULTS

Purification of rTFPI with Anticoagulant Activity Similar to That of pTFPI. Two separated fractions of TFPI were found on Mono S during the purification of heparin/Mono Q purified rTFPI (Figure 1, top). Measurement of the anticoagulant activity revealed that the peak eluting at low salt concentration (pool A) showed low anticoagulant activity as compared to chromogenic activity (ratio 0.13, Table I), while the ratio of anticoagulant to chromogenic activity was much higher for the peak eluting at high salt (pool B; ratio 1.74, Table I). A similar pattern was never observed in the purification of HeLa TFPI, which seemed to contain only the fraction with low anticoagulant activity eluting from Mono S at low salt. In the further purification by RP-HPLC both

Table I: Anticoagulant Activity of Samples from rTFPI Fractionation^a

fraction	ratio of anticoagulant to chromogenic activity
Mono S, low-salt eluate [Figure 1 (top), peak A]	0.13
Mono S, high-salt eluate [Figure 1 (top), peak B]	1.74
RP-HPLC [Figure 1 (middle), peak X]	0.08
RP-HPLC [Figure 1 (middle), peak Y]	0.12
RP-HPLC [Figure 1 (bottom), peak X]	0.92
RP-HPLC [Figure 1 (bottom), peak Y]	0.71

^aThe anticoagulant activity of the fractions was assessed in the dilute tissue thromboplastin coagulation assay described under Materials and Methods.

the rTFPI peaks from the Mono S column eluted as a double peak with an ethanol gradient (Figure 1, middle and bottom). Analysis by SDS-PAGE showed that TFPI from both Mono S peaks possessed a molecular mass just below that of the 43-kDa marker. The mass of TFPI eluting from Mono S in pool A was slightly below the mass of TFPI eluting in pool B (not shown). On SDS-PAGE it was not possible to distinguish the molecular forms that separated by RP-HPLC.

Binding properties of the TFPI forms to the Mono S cation exchanger indicated that TFPI with high anticoagulant activity has a higher positive charge than TFPI with low anticoagulant activity. Deduced from the cDNA sequence, TFPI has a very positively charged area near the C-terminus (Wun et al., 1988). Therefore, we analyzed the two forms with an antiserum raised against a peptide corresponding to this positively charged stretch of the molecule (Figure 2, left). This antiserum showed a strong reaction with the TFPI having a high anticoagulant activity but showed no reactivity against the molecular form of rTFPI that eluted by low salt from Mono S. An antiserum raised against purified rTFPI reacted equally well with the two forms of TFPI. Western blot using this antibody showed a slightly higher molecular mass of the TFPI with high anticoagulant activity.

Valentin et al. (1991) have previously demonstrated that the anticoagulant activity of partly purified plasma TFPI is very unstable. Analysis by Western blotting (Figure 2, right) demonstrated that the decrease in anticoagulant activity was followed by a slight decrease in molecular mass and reduced reactivity for the antibody against TFPI C-terminal.

Peptide Mapping of TFPI Variants. Direct structural information on Mono S separated forms of rTFPI was obtained from peptide mapping and by combination of mass spectrometry and sequence analysis. Pyridylethylated rTFPI derivatives were digested with V8 protease and subjected to RP-HPLC. Identical peptide maps were obtained for the two forms of high-activity rTFPI derivatives which had been separated and isolated by RP-HPLC prior to digestion. The peptide maps of the Mono S separated forms of rTFPI differed slightly, and Table II shows peptide data for some of the fractions obtained from low-activity TFPI (A-fractions) and from high-activity TFPI (B-fractions). The major part of the primary sequence was confirmed by mass spectrometry, and in accordance with the specificity of the V8 protease only internal cleavage after glutamic acid residues was detected. The intact C-terminal fragment was found in fraction B30 from the peptide map of high-activity rTFPI. The C-terminal amino acid is a methionine residue which by mass spectrometry was found to be partly oxidized. Furthermore, the assignment was confirmed by amino acid sequence analysis as shown in Table II. The peak corresponding to the C-terminal peptide was not present in the peptide map of low-activity rTFPI, indicating that

Table II: Analysis of Peptides Isolated from V8 Digest^a

fraction no.	mass found (Da)	mass theory (Da)	amino acid sequence	assigned peptide
A30	2019.5	2018.5	CLRAC	235-250
A31	1835.6	1833.3	nd	235-248
A34	1746.7	1746.2	CLRACK	235-247
A40	2532.3	2531.2	CLRAC	235-255
B30	880.0 [896.1]	880.1 [896.1]	EIFVKNM	270-276
B44	4342.6	4344.4	CLRACK	235-269

^aResults from mass spectrometry and amino acid sequence analysis of selected fractions from V8 protease digest of rTFPI. A-fractions were from the peptide map of low-activity TFPI, and B-fractions were from the peptide map of high-activity TFPI. Fraction A40 was found to contain a mixture of two peptides: 183-202 and 235-255. The result from a maximum of seven Edman cycles is shown. The mass in brackets for fraction B30 reflects oxidation of the C-terminal methionine residue.

Table III: Anticoagulant Activity of TFPI Isolated Directly from Cell Lines by One-Step Affinity Chromatography

cell line	ratio of anticoagulant to chromogenic activity	
	grown serum-free	grown with serum
HeLa	<0.15	<0.15
HepG2	1.4	1.5
rBHK	1.4	1.0

proteolytic degradation had occurred. Analysis by mass spectrometry identified several peptides in different fractions in the peptide map of the low-activity rTFPI, which suggests that profound heterogeneous degradation had taken place. The following C-terminal amino acids were identified: Ile247, Ser248, Gly250, and Thr255. The results were confirmed by amino acid sequencing and are shown in Table II.

Direct Immunopurification of TFPI from Cell Lines. Fermentation in serum-free medium has been demonstrated to be a cause of proteolytic fragmentation of proteins synthesized in cell culture (Kaufman et al., 1989). Since one-step affinity chromatography resulted in plasma TFPI of high anticoagulant activity, this procedure was used for the purification of TFPI from both serum-free and serum-containing culture supernatants. Culture supernatants were obtained from 100-mL T-flasks. It appears from Table III that the anticoagulant activity in the culture medium was independent of the presence of serum. Furthermore, it appears that the HeLa cells produced TFPI of low anticoagulant activity while both HepG2 and transfected BHK cells produced protein with high anticoagulant activity. Western blotting (not shown) demonstrated that TFPI from HeLa cells only showed weak reactivity for anti-C-terminus antibodies. For all preparations of TFPI, the amidolytic activity correlated with the amount of protein seen on Western blots using a polyclonal antibody toward TFPI (not shown), thus indicating that the reason for different ratios of anticoagulant to chromogenic activity was caused by variation in the anticoagulant activity.

DISCUSSION

Different procedures have been used in the purification of TFPI from cell culture media. Broze and Miletich (1987) purified TFPI from HepG2 culture medium by CdCl_2 precipitation, factor Xa affinity chromatography, gel filtration, and ion exchange. This HepG2 TFPI is of low anticoagulant activity when compared with TFPI in normal human plasma (Wun et al., 1990). We purified rTFPI from the culture media of transfected BHK cells by heparin chromatography, ion exchange, and HPLC (Pedersen et al., 1990), and it was demonstrated that also this rTFPI is of low anticoagulant activity compared with pTFPI (Nordfang et al., 1991). rTFPI purified from transfected C127 cells (Day et al., 1990) showed

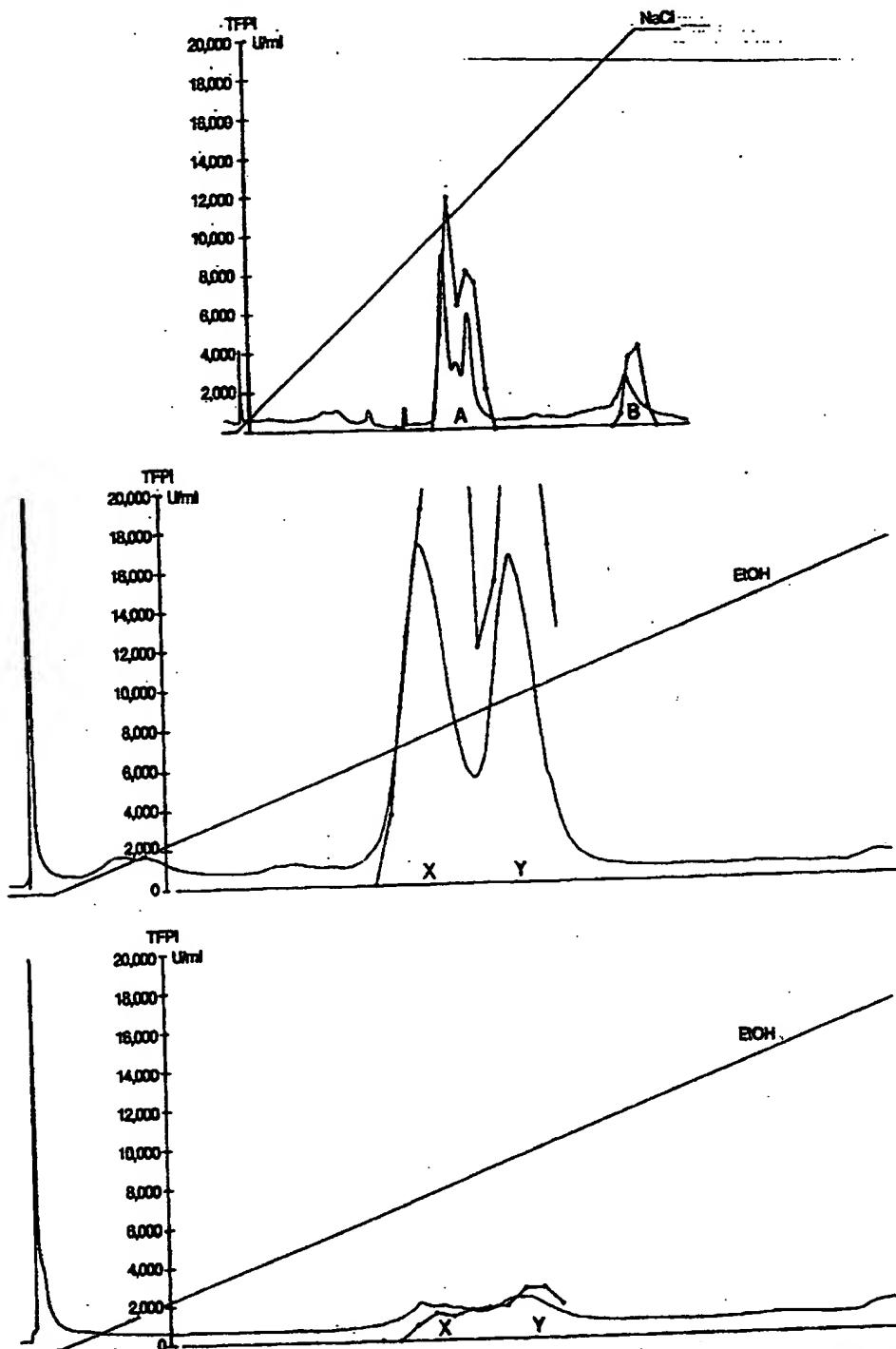


FIGURE 1: (Top) Mono-S cation exchange of heparin/Mono Q purified rTFPI. Mono Q fractions were fractionated on Mono S as described under Materials and Methods. TFPI was eluted with a gradient from 0 to 0.6 M NaCl. (Middle) RP-HPLC of low-salt eluate from Mono S. Mono S fractions (top panel, pool A) were fractionated on Pro RPC using ethanol gradient elution. (Bottom) RP-HPLC of high-salt eluate from Mono S. Mono S fractions (top panel, pool B) were fractionated on Pro RPC using gradient as above. (—) E_{280} profile; (•) TFPI chromogenic activity.

the same low anticoagulant activity (see Materials and Methods).

Using heparin affinity chromatography, we purified HepG2 TFPI with an anticoagulant activity as high as that observed for plasma TFPI (not shown). The discrepancies seemed to be dependent on fragmented TFPI present in the culture media and generated during the purification procedures. It was possible to isolate two molecular forms of rTFPI from

transfected BHK cells that differed in anticoagulant activity. By peptide mapping it was shown that the molecular form with an anticoagulant activity comparable with plasma TFPI had an intact C-terminus while heterologous C-terminal fragmentation had taken place in the low-activity form.

Using a one-step affinity chromatography procedure it was possible to obtain rTFPI of high anticoagulant activity from the transfected BHK cells, indicating that fragmentation oc-

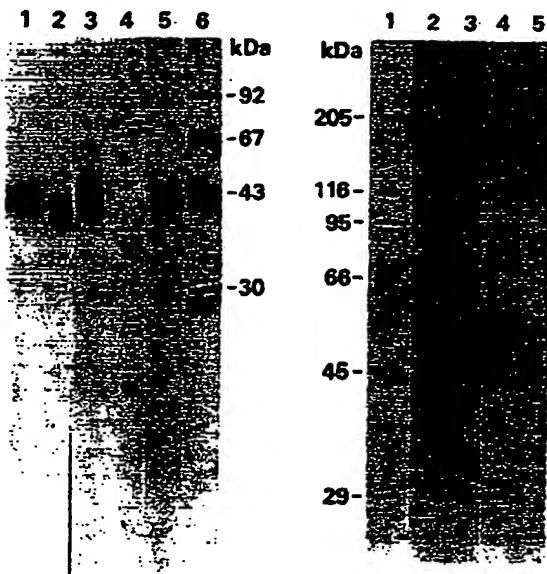


FIGURE 2: (Left) Western blot of BHK rTFPI samples developed using anti-N- and anti-C-terminus antibodies. Lanes 1, 3, and 5: 2 units of rTFPI with high anticoagulant activity [Figure 1 (bottom), peak X]. Lanes 2 and 4: 2 units of rTFPI with low anticoagulant activity [Figure 1 (middle), peak X]. Lane 6: Molecular weight markers. Lanes 5 and 6 were stained with Auro Dye. Lanes 1 and 2 were developed with polyclonal anti-TFPI antibody. Lanes 3 and 4 were developed with antibody toward the TFPI C-terminus. (Right) Western blot of plasma TFPI with decreased anticoagulant activity (7.5% separating gel). Partly purified plasma TFPI was left standing on the bench, and after 2 h at room temperature the sample was analyzed together with a sample kept at -20 °C. Lane 1: Molecular weight markers. Lanes 2 and 4: 2 units of pTFPI kept at 20 °C. Lanes 3 and 5: 2 units of pTFPI kept at -20 °C. Lane 1 was stained with Auro Dye. Lanes 2 and 3 were immunostained with polyclonal antibody. Lanes 4 and 5 were stained with anti-C-terminus antibody.

curred during purification and handling procedures. Using the same one-step affinity chromatography it was only possible to isolate TFPI of low anticoagulant activity from the HeLa cell line, and Western blotting (not shown) demonstrated that a C-terminal fragment had been cleaved from the molecule.

Upon storage, partly purified plasma TFPI loses its high anticoagulant activity. Western blotting indicated that the C-terminus of TFPI was lost concomitantly. If partly purified TFPI was added back to plasma, the anticoagulant activity was stabilized (not shown). Thus normal human plasma seems to provide inhibitors protecting TFPI from degradation. Alternatively, association to lipoproteins may protect TFPI from degradation. The association with lipoproteins does not seem to increase the anticoagulant activity of the purified TFPI since it was possible to prepare pure cell line derived TFPI with an anticoagulant activity similar to that of plasma TFPI. Western blotting showed a molecular mass of approximately 42 kDa in reducing SDS-PAGE for normal plasma TFPI, as for full length rTFPI. This is in contrast to the mass of only 36 kDa reported by Novotny et al. (1989). Recently Novotny et al. (1991) reported that heparin released TFPI differs from normal plasma TFPI since the molecular mass is 40 kDa. However, these authors used different procedures to isolate the two forms of TFPI from plasma, and our results suggest that fragmentation during purification may have caused the observed differences in molecular mass.

Structure-function studies of TFPI have concentrated on the three Kunitz inhibitory domains of the protein, and functions of other parts have been unknown so far. Here we have shown that an intact C-terminus is essential for the

activity of TFPI as measured in a coagulation assay where the total reaction time was below 2 min. Since the C-terminus is not important for activity measured in two-stage assays with long incubation times, the C-terminus may be crucial for the speed by which TFPI binds factor Xa or factor VIIa.

Presently it is not known whether a C-terminal fragmentation of TFPI, leading to a decreased anticoagulant activity, has any physiological relevance. It should be stressed, however, that all the measurements of TFPI in patient plasma samples to date have been performed using two-stage assays that do not distinguish C-terminal fragmented TFPI from intact TFPI. From these measurements it has been concluded that plasma TFPI is only moderately affected in disease states normally associated with thrombosis and only a few cases of low TFPI in connection with disseminated intravascular coagulation have been described (Bajaj et al., 1987; War et al., 1988). In situations with increased proteolytic activity, TFPI may be degraded and concomitantly the anticoagulant activity may be reduced without any detectable effect on TFPI activity measured in two-stage assays.

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